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Agonistic anti-ICAM-1 antibodies in scleroderma: Activation of endothelial pro-inflammatory cascades

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ABSTRACT

Background: Scleroderma (SSc) is a complex autoimmune disorder that can be characterised by the presence 2of circulating autoantibodies to nuclear, cytoplasmic and cell surface antigens. In particular antibodies directed against endothelial cell antigens (anti-endothelial cell antibodies; AECA) have been detected.
ICAM-1 is an adhesion molecule expressed on the surface of human endothelial cells. We have previously shown that cross-linking ICAM-1 with monoclonal antibodies leads to pro-inflammatory activation of human endothelial and vascular smooth muscle cells and that cardiac transplant recipients with transplant associated vasculopathy make antibodies directed against ICAM-1.
Objectives: To determine whether SSc patients make antibodies directed against ICAM-1 and whether these antibodies induce pro-inflammatory activation of human endothelial cells in vitro.
Methods: Using recombinant ICAM-1 as capture antigen, an ELISA was developed to measure ICAM-1 antibodies in sera from SSc patients. Antibodies were purified using ICAM-1 micro-affinity columns. HUVEC were incubated with purified anti-ICAM-1 antibodies and generation of reactive oxygen species, and expression of VCAM-1 was measured.
Results: Significantly elevated levels of anti-ICAM-1 antibodies were detected in patients with diffuse (dSSc; 10/31 32%) or limited (lSSc; 14/36 39%) scleroderma. Cross-linking of HUVEC with purified anti-ICAM-1 antibodies caused a significant increase in ROS production (2.471 ± 0.408 fold increase above untreated after 150 min $p < 0.001$), and significant increase in VCAM-1 expression ($10.6 \pm 1.77\%$ vs $4.12 \pm 1.33\%$, $p < 0.01$).
Conclusion: AECA from SSc patients target specific endothelial antigens including ICAM-1, and cause pro-inflammatory activation of human endothelial cells, suggesting that they are not only a marker of disease but that they contribute to its progression.

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1. Introduction

1.1. Scleroderma and autoantibodies

Scleroderma or systemic sclerosis (SSc), is an autoimmune connective tissue disorder that targets fibroblasts and the vascular endothelium. Limited SSc (lSSc) affects mainly the skin of the hands, face and arms, although pulmonary arterial hypertension may also be a serious complication. Diffuse SSc (dSSc) is the rapidly progressing form of the disease, characterised by severe fibrosis of large areas of skin and visceral organs, as well as widespread vascular injury (Kao and Weyand, 2010). Although the underlying pathologic triggers remain elusive, there is evidence for expansion of circulating B cells in SSc patients (Sato et al., 2004), altered T cell responses leading to a Th2 cytokine milieu (Gabrielli et al., 2007; Hasegawa et al., 1997; Hasegawa et al., 1998), and there are many reports describing the presence of autoantibodies in serum from these patients (review (Mihai and Tervaert, 2010)).

Anti-endothelial cell antibodies (AECA) were first described in the literature more than 40 years ago (review (Gabrielli et al., 2007)) in sera from patients with a number of different rheumatic diseases. AECA appear to recognise a number of different endothelial epitopes, including cell surface, cytoplasmic and nuclear antigens, and there is increasing evidence that at least some species may have agonistic properties i.e. they appear to cause endothelial cell activation and therefore are hypothesised to contribute to disease progression.

1.2. Agonistic anti-ICAM-1 antibodies

ICAM-1 is a 90 kDa Ig superfamily protein, expressed on the surface of several cell types including endothelial cells, where it has been

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shown to be a critical molecule for firm adhesion and trans-endothelial migration of several leukocyte subsets. ICAM-1 itself can activate cell signalling cascades after receptor multimerisation which can be achieved in vitro by co-culturing of EC with LFA-1 positive leukocytes, artificial clustering of ICAM-1 on EC by fibrinogen, or cross-linking with anti-ICAM-1 antibodies (review (Hubbard and Rothlein, 2000; Lawson and Wolf, 2009)).

A number of signalling molecules and adapter proteins have been linked with the ICAM-1 signalling cascade in vitro, depending on cell lineage or origin of vascular bed of EC used, and the experimental model (for review see (Lawson and Wolf, 2009)). Cross-linking of ICAM-1 on the cell surface of endothelial cells leads to its redistribution from the detergent soluble to insoluble fraction, suggesting that it is involved with endothelial cytoskeletal rearrangements required for leukocyte emigration (Amos et al., 2001). A number of studies have also demonstrated that ICAM-1 cross-linking, either with monoclonal antibodies or during co-culture of endothelial cells with T cells, leads to activation of molecules involved in these rearrangements including Rho-A, a small GTPase responsible for actin stress fibre formation (for review see (Lawson and Wolf, 2009)).

In addition to its role in actin cytoskeleton rearrangements, cross-linking of ICAM-1 with monoclonal antibodies has also been shown to activate pro-inflammatory cascades, via activation of MAPK kinases ERK-1/2 and/or JNK (Etienne et al., 1998; Lawson et al., 1999; Sano et al., 1998). Activation of ERK-1 leads to AP-1 activation (Lawson et al., 1999) and ERK-dependent production and secretion of IL-8 and RANTES (Sano et al., 1998), expression of VCAM-1 expression on the cell surface (Lawson et al., 1999; Lawson et al., 2001). ICAM-1 cross-linking also upregulates tissue factor production (Schmid et al., 1995) and expression of proinflammatory cytokines including IL-1β (Koyama et al., 1996).

We have previously identified agonistic anti-ICAM-1 antibodies in serum from cardiac transplant recipients with chronic transplant vasculopathy and shown that serum from these patients can activate phosphorylation of p42/p44 Erk MAPK (Lawson et al., 2005). In the present study we have identified anti-ICAM-1 antibodies present in serum from patients with SSc significantly above levels seen in healthy volunteers. Using microaffinity columns we have purified IgG anti-ICAM-1 antibodies from these patients and shown that they bind to human umbilical vein endothelial cells (HUVEC) and activate reactive oxygen species production and VCAM-1 expression.

2. Methods

2.1. Materials

All chemicals were from Sigma (Poole, Dorset, UK) unless otherwise stated. All plastics and tissue culture plates and flasks were from Nunc (Fisher Scientific, Loughborough, UK). All tissue culture consumables were from PAA (UK) except for endothelial growth supplement (ECGS) from Sigma. Anti-VCAM-1 MAb clone 1.4C3 and anti-ICAM-1 MAb clone 6.5B5 were gifts from Professor Dorian Haskard (Imperial College, London). Secondary FITC or HRP conjugated antibodies were from DAKO. Rabbit anti-mouse Ig (RAM) used to cross-link 6.5B5 was from DAKO. Rabbit anti-human Ig (RAH) used to cross-link patient antibodies was from Jackson Immunotech (Stratech, Luton, UK). Recombinant human TNF was from Insight Biotechnology (Wembley, London, UK).

2.2. Source of human sera

Sera were collected from 60 patients with scleroderma (n = 32 lSSc, n = 28 dSSc) and from 26 healthy controls. Sera were collected at the Royal Free Hospital; Centre for Rheumatology, London, UK; with the informed consent of the patient. Volunteers with SSc fulfilled the criteria of the ACR for the diagnosis of SSc and ethical approval was obtained from the Royal Free Hospital ethical practices sub-committee.

2.3. Preparation of recombinant ICAM-1

Recombinant human ICAM-1 was cloned into pET15b (Novagen, MerckMillipore, Darmstadt, Germany) and purified from cultures of BL21 *E. coli* using His-bind columns as described previously (Holder et al., 2008).

2.4. ELISA

Purified recombinant human ICAM-1 (rhICAM-1) was coated onto alternate rows of 96 well Maxisorp ELISA plates (Nunc, Fisher Scientific, Loughborough, UK) at a concentration of 2 μg per well in 150 μl 1 × PBS. The remaining wells were coated with 1 × PBS alone to measure non-specific binding of Ig to the microtitre plate. Plates were incubated at 4 °C overnight. The following morning plates were washed 3 times in 1 × PBS 0.1% Tween-20 (PBST) and all wells were blocked for 1 h with 300 μl 5% non-fat milk powder PBS-T (Marvel-PBST), followed by three washes in PBST. Patient samples were diluted 1:50 in Marvel-PBST and added to rhICAM-1 coated and PBS coated wells in triplicate at 100 μl/well. Samples were incubated for 2 h at room temperature followed by three washes in PBST. Positive controls were monoclonal anti-ICAM-1 6.5B5 at 10, 5, 2.5, 1.25 μg/ml (10 μg/ml was used for calculation of ELISA RATIO, see below) and negative controls were Marvel-PBST alone. Wells were incubated with 100 μl HRP-conjugated rabbit-anti-human-IgG or IgM or HRP-conjugated-rabbit-anti-mouse IgG as appropriate and incubated for 45 min at room temperature, followed by three washes in PBST. 100 μl TMB supersensitive substrate (Sigma) was added and plates were incubated for 5 min at room temperature before the reaction was stopped by addition of 50 μl 1 M H_2SO_4. Plates were read using a Wallac 1410 plate reader at 450 nm. In order to normalise OD results across multiple plates and with multiple batches of recombinant ICAM-1, an "ELISA ratio" (ER) was used which was calculated according to the equation:

$$ER = \frac{[OD_{450}\,\mathrm{serum\,Ig\,bound\,to\,ICAM1\,coated\,wells}] - [OD_{450}\,\mathrm{serum\,Ig\,bound\,to\,PBS\,coated\,wells}]}{[OD_{450}\,6.5B5\,(10\ \mu g/ml)\,\mathrm{to\,ICAM1\,coated\,wells}] - [OD_{450}\,6.5B5(10\ \mu g/ml)\,\mathrm{bound\,to\,PBS\,coated\,wells}]}.$$

Sera were deemed to contain a high titre of anti-ICAM-1 antibodies if the ER was greater the mean ER + 3sd of the negative control sera.

2.5. Purification of ICAM-1 antibodies from human serum

Recombinant hICAM-1 was prepared as described above and coupled to Pierce micro-affinity columns according to the manufacturer's instructions (Fisher Scientific). Anti-ICAM-1 antibodies were purified from 200 μl SSc patient serum (n = 6 patients) selected as highly positive for anti-ICAM-1 as determined by our ELISA, and the manufacturer's instructions were followed. All washes were kept for use as negative controls. Purified antibodies were analysed using the anti-ICAM-1 ELISA described above to determine the relative concentrations compared to the 6.5B5 positive control anti-ICAM-1 MAb, to confirm their presence (data not shown).

2.6. HUVEC isolation and culture

Human umbilical cords were collected from the Royal London Hospital with ethical approval from the East London Research Ethics Committee. Human umbilical vein endothelial cells (HUVEC) were

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